Cellulolytic bacteria as potential farm residue degraders

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "**Cellulolytic bacteria as potential farm residue degraders**" is based on the results of investigations carried out by me in the Microbiology Programme at the School of Biological Sciences and Biotechnology, Goa University under the Supervision of Prof. Sandeep Garg and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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COMPLETION CERTIFICATE

This is to certify that the dissertation report **"Cellulolytic bacteria as potential farm residue degraders"** is a bonafide work carried out by **Ms. Tejasvi Vasu Zalmi** under my supervision/mentorship in partial fulfilment of the requirements for the award of the degree of Master of Science in Microbiology, in the Discipline Microbiology, at the School of Biological Sciences and Biotechnology, Goa University.

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PREFACE

In recent years, the management of farm residues has emerged as a critical environmental concern. Agricultural waste management methods such as in field decomposition or burning have led to significant air and soil pollution. As the global population continues to grow, so does the demand for food production leads to an increase in agricultural waste However, utilizatioin of microbial consortia of cellulolytic bacteria as potential degraders of farm residues can holds significant approach and as a way for effective and eco-friendly waste managing practices. Harnessing the enzymatic prowess of lignolytic enzymes produced by these bacteria presents an opportunity to convert recalcitrant farm residues into valuable resources, reducing environmental pollution and enhancing soil health.

This research aims to contribute to the development of sustainable solutions for agricultural waste and bioresource utilization.

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LIST OF TABLES

ABBREVIATIONS USED

Abbreviation	Expansion
CMC	Carboxymethyl Cellulose
CR	Crop Residues
GPP	Glucose phosphate broth
TSI	Triple Sugar Iron

ABSTRACT

Lignocellulosic biomass has become an increasing concern in recent years which plays a major role in the economic production of various value-added products and biofuels. Cellulolytic bacteria break down the cellulose aiding in decomposition process and recycling of organic matter in the ecosystem. In this study, bacteria were isolated and screened for the production of enzymes involved in cellulosic biomass degradation. The activity of three enzymes were examined using plate screen methods. Among thirty-seven isolates, ten isolates showed the ability to degrade Carboxymethyl cellulose. Seven isolates showed amylase activity and six isolates were able to show the production of xylanase. Enzyme producing isolates were also tested for acid production. Enzyme showing high activity can be used for the formation of microbial consortia to effectively degrade farm residues by employing optimized parameters. Industrially important enzymes have diverse applications including beverage production, pharmaceuticals, paper and pulp industry, biofuels and in food additives. Successful development of this technology could offer a sustainable solution for lignocellulosic waste management.

KEYWORDS: Cellulose, Farm residues**,** Carboxymethyl cellulose (CMC)**,** Cellulolytic bacteria**,** Enzymes

1. INTRODUCTION

Lignocellulosic waste consists of organic matter primarily composed of three structural polymer- Cellulose, hemicellulose, and lignin. These compounds provide structural support to the plants and comprises a major portion of its cell. Lignocellulosic waste derives from various sources such as agricultural residues, forestry residues and municipal solid waste and represents a significant renewable biomass resource. This waste has gained increasing attention for its potential in biofuel production, composting and other sustainable practices due to its abundance and potential for conversion into various useful products.

Lignocellulosic crop residues are utilized for both domestic and industrial needs yet a considerable portion often remains unutilized and is either dumped in fields or burnt (Gupta et al.,2017). The proper disposal of these residues poses a significant challenge, particularly for farmers in developing countries. Despite the availability of alternative disposal methods, burning is often preferred due to its rapid and cost-effective nature, facilitating quick field clearance and land preparation for subsequent crops. However, this practice contributes to the emission of various air pollutants including Carbon dioxide, methane, ammonia, and particulate matter, posing environmental and health concerns (Mittal et al.,2009; Zhang et al., 2011).

Cellulose constitutes the most abundant, renewable polymer resource available today worldwide. It is the largest reservoir of organic carbon on the Earth. Cellulose and its derivatives are used in pharmaceuticals, medicinal purposes, food additives, building supplies and clothing purpose. Converting cellulose from energy crops into biofuels such as cellulosic ethanol is under exploration as an alternative fuel source (Sundarraj et al.,2018). Overall, the conversion of cellulose into valuable byproducts represents a sustainable solution for waste management and resource conservation.

1.1. HYPOTHESIS:

1.Utilizing cellulolytic bacteria for farm residue degradation can have ability of natural decomposition

2.Reduction of stubble burning

1.2.AIM

To isolate and screen for bacteria and application of their consortium in farm residue degradation.

1.3. OBJECTIVES:

- Isolation and screening of cellulase-producing bacteria
- Secondary screening for other enzymes (xylanase, amylase)
- Segregation based on enzymes they produced
- Application of consortium for farm residue degradation.

2.LITERATURE REVIEW

2.1 Farm residues and its management

Farm residues also known as agricultural residues, are the organic byproducts remaining after agricultural activities. India, the second largest agro-based economy with year-round crop cultivation, generates a large amount of agricultural waste, including crop residues. These crop residues like stalks, leaves and husks left in fields after harvest, as well as animal manure and agricultural processing byproducts. Recycling crop residues offers the advantage of converting surplus residues into valuable products to fulfil nutrient requirements for soil microorganisms and subsequent crops (Maurya et al.,2020). Farm residues play a crucial role in soil health and fertility by providing organic matter, moisture retention and nutrient availability, ultimately improving crop productivity. Additionally, farm residues can be utilized for bioenergy production through processes like anaerobic digestion and combustion, contributing to renewable energy generation and reducing greenhouse gas emissions. Moreover, their incorporation into animal feed or as bedding material provides a sustainable way to recycle nutrients within the agricultural system, minimizing waste and promoting circularity. Efficient management of farm residues is essential for sustainable agriculture and reducing environmental impacts.

2.1.1. Composition of farm residues

The constituents found in residues and their susceptibility to microbial breakdown can be categorized into six main groups:(1) cellulose, constituting approximately 15-60% of the dry residue weight; (2) hemicelluloses (10-30%); (3) lignin (5-30%);water-soluble sugars, amino acids and aliphatic acids (5-30%); (5) fats, oils, waxes and pigments soluble in ether and methanol; and (6) proteins containing nitrogen and sulfur. Among these, cellulose, hemicellulose and proteins are readily degraded by microbial action (Mohammed et al.,2018).

a) Cellulose

Cellulose is a fibrous, crystalline polysaccharide composed of repeated D- glucose units joined by β-1,4 glycosidic linkage (Zaghoud et al.,2019). This polymer has biodegradable, biocompatible and renewable properties. Plants produce 4×109 Tons of cellulose annually. There has been a growing interest in using cellulose, a major waste product from agricultural work, as feed and an energy source in the form of stalks, stems, and husks (Balachandrababu et al., 2012)

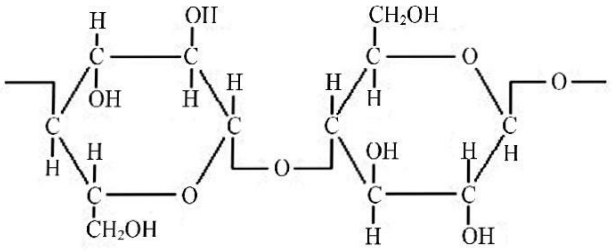

Fig 2.1.1: Structure of cellulose (Richards et al., 2012)

Cellulose molecules contain two types of hydrogen bonds, facilitating tight packing into crystallites. These bonds hinder water and enzyme penetration, except for exoglucanase, which degrades the terminal glucosidic bond. Crystalline cellulose, due to its tight structure, is insoluble and requires exoglucanase for degradation, while amorphous cellulose allows penetration of endoglucanase, leading to faster hydrolysis.

b) Hemicellulose

Hemicellulose, is a heterogeneous, containing sugars and uronic acids. It is amorphous, short chained and capable of joining lignin and cellulose together (Chundawat et al., 2010). It contains several heteropolymers such as glucomannan, galactomannan, xylan,

glucuronoxylan, xyloglucan and arabinoxylan (Isikgor et al.,2015). The composition of hemicellulose varies depending on the source of the lignocellulose.

c) Lignin

Lignin, constituting 15-30% of lignocellulosic biomass which is a nature's most abundant source of renewable aromatic carbon. Comprising phenylpropane units with various linkages β-O-4, α-O-4,4-O-5, β-β, β-5, 5-5, β-1, it is challenging to degrade due to its high molecular weight, complex structure, and limited solubility, leading to adverse environmental effects(Zhang et al., 2021).

2.1.2. CELLULOSE DEGRADING MICROORGANISMS

Cellulolytic enzymes are synthesized by various microorganisms, with fungi and bacteria being the primary agents of cellulose degradation. These microorganisms include aerobic and anaerobic mesophilic bacteria, filamentous fungi, thermophilic and alkaliphilic bacteria, actinomycetes, and certain protozoa. However, fungi are particularly renowned for decomposing organic matter, especially cellulose-rich substrates (Lynd et al., 2002).

Microorganisms tackle cellulose degradation using a multi-enzyme system. Aerobic bacteria produce numerous individual and extracellular enzymes with binding modules for different cellulose conformations, while anaerobic bacteria possess a unique extracellular multi-enzyme complex called cellulase. The primary cellulose utilizing species are aerobic and anaerobic hemophilic bacteria, filamentous fungi, basidiomycetes, thermophilic bacteria, and actinomycetes (Wright et al., 2003).

Certain types of bacteria such as *Clostridium, Cellulomonas, Cellulosimicrobium, Thermomonospora, Bacillus, Ruminococcus, Erwinia, Bacteroides, Acetovibrio, Streptomyces, Microbispora, Fibrobacter, and Paenibacillus* have the ability to produce cellulases(Bautista-Cruz,et al.,2024).

Initially, microorganisms responsible for cellulose decomposition catalyze an enzymatic hydrolysis of the complex polymer, collectively known as cellulase. Although fungi exhibit efficient cellulase activities, there's growing interest in cellulase production by bacteria due to their higher growth rates and potential for economic efficiency. Finding a novel bacterial strain with hyper cellulase productivity, enhanced activity, and high stability against temperature, pH, and non-aseptic conditions could greatly benefit the process. Cellulase was first discovered in 1983 from the anaerobic, thermophilic spore-forming Clostridium thermocellum (Maki et al., 2011).

2.1.3. FUNCTIONS OF CELLULOLYTIC BACTERIA IN AGRICULTURE FARM RESIDUE DEGARDATION

Cellulolytic bacteria play crucial roles in agriculture farm residue degradation by breaking down cellulose, the main component of plant cell walls, into simpler compounds. Their functions include:

1. Cellulose Degradation: Cellulolytic bacteria produce enzymes such as cellulases, which break down cellulose into glucose and other simple sugars, which can then be utilized by other microorganisms or plants.
2. Nutrient Cycling: By decomposing plant residues, cellulolytic bacteria release nutrients like nitrogen, phosphorus, and potassium back into the soil, making them available for plant uptake, thus promoting soil fertility
3. Soil Structure Improvement: The activity of cellulolytic bacteria helps to enhance soil structure by increasing soil porosity and aggregation, which improves water infiltration and retention.

4. Organic Matter Decomposition: They contribute to the decomposition of organic matter in the soil, leading to the formation of humus, which improves soil fertility and helps to retain moisture.
5. Carbon Sequestration: Cellulolytic bacteria play a role in carbon sequestration by incorporating carbon from plant residues into the soil organic matter, thus helping to mitigate climate change.

2.1.4. Methods for crop residues utilization

Crop residues generated from agricultural activities can be employed through various methods and many developing countries are utilizing all the residue produced for some or other purpose (Bhuvaneshwari et al.,2019).The use of crop residues are as follows:

a) Livestock feed

In many developing nations, a significant portion of crop residues serves as livestock feed (Devi et al., 2017). Ruminants can digest cellulose due to enzymes produced by rumen microorganisms (Van Soest, 1994). However, the presence of lignin reduces overall digestibility. Crop residues, like cereal straw, particularly rice straw, are low in protein and high in crude fiber (Goswami et al., 2020). Conversely, leguminous crop residues offer better crude protein and nutrient content (Win et al., 2021). Common practices include stubble-grazing and minimal processing of residues, often made more nutritious with nitrogen-rich additives. Western countries often use crop residues for bedding, notably wheat straw, whereas in developing countries, they're valued as low-cost animal feed during fodder shortages (Goswami et al., 2020).

b) Bedding material

Crop residues, particularly straw, serve as bedding materials for animals due to their exceptional water absorption capabilities (Lips et al., 2009), ensuring animal cleanliness and comfort (Werhahn et al., 2010). This practice simplifies waste collection, such as dung and droppings. Various residues like rice hulls, straw, and peanut hulls are utilized in poultry bedding to maintain bird health and dryness (Grimes et al., 2002). Placing crop residues in animal shelters enhances their compost degradability (Tait et al., 2009), producing nitrogen-rich litter that yields high-quality compost (Duan et al., 2021). This method not only facilitates animal care but also optimizes crop residue utilization. Recent studies by Duan et al. (2021) highlight that combining bedding material with cow manure accelerates composting, particularly with a ratio of 40:60, significantly enhancing organic matter degradation and composting temperatures.

c)Mushroom cultivation

Wheat and rice straws serve as ideal substrates for cultivating button mushrooms (Niazi et al., 2021) and straw mushrooms (Biswas and Layak, 2014), among the most commonly cultivated mushroom varieties. Conversely, logs and stumps are preferred for growing Lentinus and Pleurotus mushrooms. To optimize button mushroom growth on straw, it's often mixed with manure and hay (Wuest et al., 1987). Mixing paddy straw with banana pseudo stem in a 50:50 ratio enhances mushroom yield, with hand-threshed rice straw proving more effective than machine-threshed straw (Biswas and Layak, 2014). Outdoor cultivation of Volvariella simply requires wetted straw, but indoor cultivation benefits from mixing rice straw with other crop residues for improved yield (Hamlyn, 1989). Utilizing crop residues for mushroom cultivation presents a profitable avenue

within diversified agriculture, transforming crop waste into high-value, nutrient-rich food sources (Hu et al., 2021)

d)Building materials

Use of cereal crops residue for roofing of houses and sheds is also a common practice in many countries. Making bricks and walls from straw-clay mixtures is an ancient technique where straw is used to give strength to the material. Modern applications of straw with a suitable binder are in making boards, bio-composite materials, etc., for interior partitioning, packaging, false roofing and other related applications (Mo et al., 2005). Attempts of making boards from straw without using a bonding material is also giving good results (Zhao et al., 2014). There is an increasing demand for the environmentally friendly composite boards, more particularly, as a replacement of synthetic materials and solid wood material for use in the packaging, aircraft, furniture, and automobile industry (Aladejana et al., 2020). Abundantly available natural fibres in the form of residues of wheat, rice, corn, etc. have a great potential to be utilized to produce these new materials.

e) Crop residues as biochar

Crop residues can be converted into biochar through pyrolysis, a process involving high-temperature burning in limited oxygen (Singh and Sidhu, 2014). Biochar, resistant to decomposition, can persist in soil for centuries, offering a means of sequestering carbon and reducing agriculture's carbon footprint (Puget and Lal, 2005). Its application as a soil amendment enhances fertility, soil organic carbon, and reduces greenhouse gas emissions, as well as improves fertilizer efficiency by reducing leaching (Chan et al., 2007).

2.1.5. On farm utilization of crop residues

To return and recycle all the nutrients from the Crop Residues(CR) in the, farm its utilization must be done in the farm itself, which will also save time and drudgery in its transportation.

a) In situ incorporation

Incorporating crop residues (CR) into soil on-site involves mixing straw, stubble, or leftover crop remnants with topsoil during tillage practices. Studies have demonstrated that incorporating rice residues and weed biomass with Trichoderma viridi inoculum improves soil properties and crop yields in rice and toria systems over three years (Choudhary et al., 2020). Factors like CR composition, soil type, seasonal variations, and microbial activity influence decomposition rates, leading to varied decomposition periods (Jat et al., 2017). Smaller particles degrade faster due to increased surface area but may face inhibition from phenolic substances (Fox et al., 1990).

b) Ex-situ crop residue composting

On-farm utilization of crop residues through composting offers a sustainable solution for recycling nutrients. By decomposing crop residues aerobically or anaerobically with farmyard manure, nutrient-rich compost is produced, aiding soil health and crop productivity. To accelerate decomposition, pretreatment with lignocellulose-degrading microbes can be employed. Composting, though involving manual labor, requires no additional capital or machinery. Mixing crop residues with manure enhances compost quality and decomposition rates (Hubbe et al.,2010). Compost tea, derived from this compost, presents a water-based, nutrient-rich solution promoting plant growth and disease resistance. These

practices not only reduce dependency on chemical inputs but also offer economic benefits, particularly for small-scale farmers, contributing to sustainable agriculture.

2.1.6. Industrially important enzymes

Industries heavily depend on microbial enzymes to transform agricultural waste into valuable byproducts (Nayak et al., 2019). Enzymes can be harnessed by many industries such as detergents, textiles, and others for commercial use. Economically important hydrolytic enzymes include amylase, cellulase and xylanase.

a) Cellulase

Cellulose is the most abundant bioresource found in biosphere. Cellulase represents a vital group of enzymes responsible for the breakdown of cellulose into soluble sugar (Pal K.et al.,2021). These sugars can then be used to produce bioethanol, a renewable fuel source. (Bhardwaj et al.,2021). Additionally, cellulase is used in various industries such as textile sector for biofinishing processes like bio polishing, as well as in household laundry detergents, enhancing fabric softness. Cellulase-producing bacteria (CPB) have been isolated from various different sources including compost, soil, organic matter, plant and other cellulose rich sources. Carboxymethyl cellulose (CMC)is a substrate used for cellulase enzyme.

Examples of CPB are *Bacillus polymixa, B. subtilis,Paenibacillus campinasensis* and *B.cereus,Cellulomonas cellulans,B.licheniformis,Bosea* sp. *and B.thuringiensis*(Sadhu and Maiti,2013).

Cellulases are classified into three categories: β-glucosidase, endo-1,4-β-D-glucanase (endoglucanase) and exo-1,4-β-D-glucanase (exoglucanase). The function of these enzymes are:

Exoglucanase: It forms glucose or cellobiose as the major end product by hydrolyzing non reducing ends of crystalline cellulose.

Endoglucanase: It carries hydrolysis of the internal sites of oligosaccharides present in amorphous cellulose, carboxyl methyl cellulose and cellooligosaccharides

B-glucosidase: It catalyzes hydrolysis of cellobiose and cellodextrin at the non reducing end.

A synergitic action of above mentioned three enzymes are required for achieving the effective degradation of cellulose (Ejaz et al.,2021)

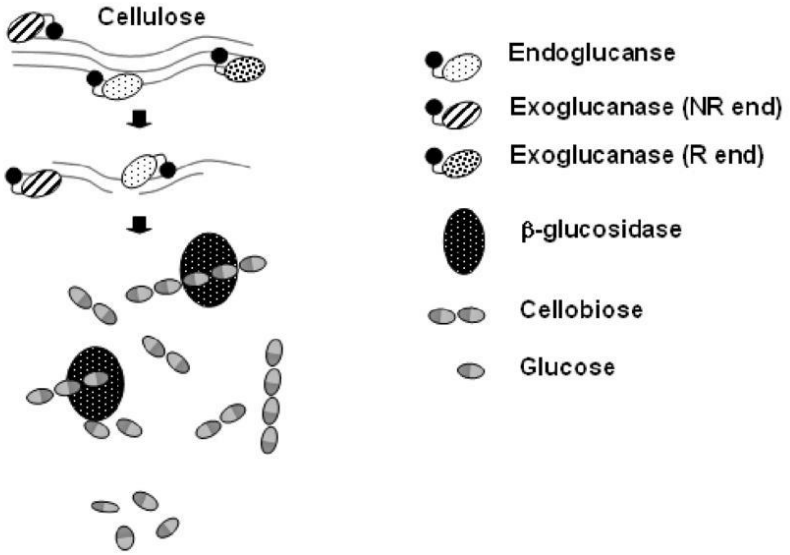

Fig 2.1.6.1: Mechanism of cellulase action(Mathew et al.,2008)

b.Xylanase

D-xylan, a polysaccharide prevalent in both hardwoods and annual plants, constitutes a significant portion, ranging from 20-35% of their total dry weight, surpassing even cellulose. Softwoods, on the other hand, contain a lower proportion, about 8% of dry weight. Structurally, xylan consists of short linear chains of beta-D-xylopyranosyl residues linked by (1-4) bonds. The enzyme xylanase is pivotal in breaking down xylan, operating alongside various hydrolytic enzymes such as endo-B-1,4-xylanases and beta-xylosidases. Endo-B-1,4-xylanases target the primary chain of xylan, while beta-xylosidases cleave xylooligosaccharides into D-xylose subunits. In addition to these enzymes, several accessory enzymes participate in xylan degradation(Poutanen et al.,1991).

The applications of xylanase, particularly in tandem with cellulose, are diverse, primarily revolving around the bioconversion of lignocellulosic materials derived from agricultural and forestry waste. Despite xylan's susceptibility to acid hydrolysis, enzymatic degradation remains a viable alternative. Notably, xylanase finds specific utility in producing oligosaccharides from plant-derived xylan, a process successfully commercialized in Japan. The resulting oligosaccharides, primarily xylobiose and xylotriose, serve as valuable food additives and artificial sweeteners.

Furthermore, xylanase demonstrates potential in various other applications, including enhancing starch recovery from wheat flours, facilitating fruit juice extraction and clarification, modifying baking products, and improving the digestibility of animal feed, thereby enhancing feed efficiency (Watson et al.,1993).

c.Amylase

Starch is most significant carbon reserve on the planet and has an immense commercial importance (Martin and Smith, 1995). It comprises glucose polymers forming a semi-crystalline structure known as starch granules. The breakdown of starch molecules is facilitated by amylase enzymes, which come in two sub-types: alpha and beta amylases. These enzymes cleave α-1-4 glycosidic bonds within the starch molecule. Industries prefer bacterial amylase due to its cost-effectiveness, eco-friendliness, and ability to produce high-quality and high quantities. Bacillus species, including *B. subtilis, B. megaterium, B. vulgaris, B. licheniformis, B. amyloliquefaciens*, and *B. cereus*, are major producers of bacterial amylase (Gopinath et al., 2017). Additionally, species from genera such as *Chromohalobacter, Corynebacterium*, *Lactobacillus*, and *Pseudomonas* are known to produce amylase. Notably, halophilic bacteria and archaea such as *Haloarcula hispanica, Halobacillus sp., Chromohalobacter sp., Bacillus dipsosauri,* and *Halomonas meridiana* also exhibit amylase production (Kathiresan and Manivannan, 2006). Amylase constitutes up to 25% of the total world enzyme production, making it one of the most abundant enzymes globally (Souza and Magalhães, 2010). Its applications span various industries including detergents, food, paper, textiles, and pharmaceuticals. Moreover, it plays a crucial role in the production of syrups derived from corn, maltose, and glucose (Mojsov, 2012).

2.1.7. Microbial consortia

Microbial consortia are communities of diverse microorganisms that work together synergistically to break down complex organic matter. The use of microbial consortium on crop residues can speed up their breakdown, bring more help.

Although microbial consortia hold promising benefits for farm residue degradation, their use is still at an early stage and demands additional research and development. The main difficulties include optimizing consortia composition for different kinds of residue, figuring out how different types of microorganisms interact, and making enough of the microbial consortia for use on farms.

Research has explored the practicality of a composting system using bio-inoculants like *Pleurotus sajor-caju, Trichoderma harzianum, Aspergillus niger*, and *Azotobacter chroococcum*, followed by vermicomposting of wheat straw(Singh et al.,2002).This approach has led to faster degradation of lignocellulosic waste with significant reductions in cellulose, hemicellulose and lignin content.

Another study focused on speeding up sugarcane residue decomposition by employing a fungal-bacterial consortium consisting of *Cellulomonas sp.,* and *Azospirillum brasilense* (Beary et al.,2002).Results demonstrated that applying the consortium to the residue, mixed with soil and supplemented with 0.3% molasses, significantly accelerated decomposition. Adding microbial culture alongside bulking agents and nutrient sources can enhance the degradation process and promote microbial growth and survival in natural conditions.

3.MATERIALS AND METHODS

3.1. Sample collection

The samples for isolation of cellulose degrading bacteria were collected from various samples having high cellulose content such as coconut husk, leaf litter, rice straw, sugarcane bagasse, grass, cellulose rich kitchen waste, garden soil and termites.

Samples were collected in a sterile zip-lock bag and brought to the laboratory.

3.1 Enrichment

A Carboxymethyl cellulose broth (Appendix I) was prepared in a sterile flask that support the growth of cellulose-degrading bacteria and the pH of the broth was adjusted to the optimal range of 6.8-7.2. (Rawway et al.,2018). The broth was inoculated with samples. Inoculated broth with samples was incubated at room temperature (28^0 C) for 5-6 days to allow the growth of bacteria capable of cellulose degradation.

3.2. Isolation of cellulolytic bacteria

For the isolation of cellulose degrading bacteria,0.1 ml of the enriched broth was taken into the pipette and transferred on the basal medium containing CMC in an aseptic condition. The sample was spread plated evenly on the surface with the help of a spreader. Inoculated CMC agar plates were kept inverted and incubated at room temperature (28^0 C) for 48 hours. Bacterial colonies obtained after incubation were streaked on CMC agar plates in an aseptic condition to attain isolated colonies and was further incubated at room temperature (28^0 C) for 48 hours.

3.3. Subculturing and maintenance of isolated bacterial colonies

After purification process, the isolates were maintained in an active state by subculturing routinely on CMC agar plate. Culture plates containing isolated colonies were stored in a refrigerator to preserve the culture for future use.

3.4. Primary screening of cellulase enzyme

For determining the cellulase activity of the bacterial isolates, the isolates were spot inoculated on the basal medium (Appendix I) supplemented with 1% CMC as substrate. The pH of the media was adjusted to 6.8-7.2. Plates were incubated at room temperature(28^0C) for 48 hours.

After incubation, the plates were flooded with 0.1% Congo Red solution and kept for 15-20 minutes. The stain was removed by pouring off and destained the plates by using NaCL solution. After destaining, the plates were observed for yellow opaque zones around the bacterial colonies which indicates the hydrolysis of cellulose.

3.5. Secondary screening of enzymes:

3.5.1. Xylanase activity

For determination of xylanase activity, the isolates were spot inoculated on the basal medium supplemented with 1% xylan (birchwood) and the plates were incubated at room temperature(28^oC) for 48 hours. After incubation, the plates were flooded with 0.1% Congo red solution and kept for 15-20 minutes. After staining, the dye was removed by pouring off and the plates were destained with NACL solution. Yellow opaque zones were observed around the colonies indicates the hydrolysis of Xylan.

3.5.2. Amylase activity

For determination of amylase activity, basal medium was supplemented with 0.5% starch. Isolates were spot inoculated on solidified medium and the plates were incubated at room temperature (28^{o} C) for 48 hours. After incubation, the plates were flooded with Lugol's iodine solution to observe degradation of starch, Zone of clearance around the colony indicates hydrolysis of starch.

3.6. Screening of isolates for acid production

Glucose Phosphate Broth(Appendix II) was prepared in an aseptic condition according to the composition. The bacterial isolates were inoculated in broth. Uninoculated tube was kept as control. Tubes were incubated at room temperature for 24-48 hours. After incubation, the isolates were checked for acid production by adding few drops of methyl red indicator to culture broth. Tubes were mixed gently by shaking to ensure thorough mixing of the indicator. Methyl red will turn red at a pH below 4.4. Observed the colour change of the broth. A red colour indicates a positive result that the bacteria produce acidic end products from glucose fermentation. A negative result was indicated by yellow or orange colour.

3.7. Characterization and identification of the bacterial isolates

The isolates showing above enzyme activities were identified by Gram staining, colony characteristics and biochemical tests.

3.7.1. Gram staining

A clean grease free glass slide was taken. A drop of sterile saline was put on a slide. Isolated bacterial colonies were picked using loop and was transferred in a saline for making a bacterial smear. Smear was prepared, dried and heat fixed on the burner. Primary stain (crystal violet) was added to the bacterial smear made on a slide. Stain was allowed to remain for 1 minute Then the stain was removed by pouring off and the slide was flooded with Gram's iodine (Appendix II) for 1 minute. The smear was then decolourized with 70% ethanol for 1 minute. The slide was counterstained with saffranine (Appendix II) for 30 seconds. It was washed and air dried. Observed under oil immersion objective.

3.7.2. Biochemical tests

3.7.2.1. Indole test:

A sterile tryptone broth (Appendix was prepared in an aseptic condition. The 5 ml of the broth was distributed in sterile test tubes. The bacterial colonies were then inoculated aseptically in a tryptone broth. Uninoculated tube was kept as control. The tubes were incubated for 24 hours. After incubation,0.5 ml of Kovac's reagent was added to the broth. A positive test was indicated by the formation of red colour ring which shows that bacteria present in a medium is capable of producing indole. Negative test was indicated by no formation of red colour ring indicating that the bacteria being tested does not produce indole from tryptophan

3.7.2.2. Methyl red test

Glucose Phosphate Broth(Appendix II) was prepared in an aseptic condition according to the composition. The bacterial isolates were inoculated in broth.

Uninoculated tube was kept as control. Tubes were incubated at room temperature for 24-48 hours. After incubation, the isolates were checked for acid production by adding few drops of methyl red indicator to culture broth. Tubes were mixed gently by shaking to ensure thorough mixing of the indicator. Methyl red will turn red at a pH below 4.4. Observed the colour change of the broth. A red color indicates a positive result that the bacteria produces acidic end products from glucose fermentation. A negative result was indicated by yellow or orange colour.

3.7.2.3. Voges-Proskauer test

The bacterial isolates were inoculated in glucose phosphate broth (MR-VP broth) aseptically using a sterile inoculating loop. Uninoculated tube was kept as control and the tubes were incubated for 48 hours. After incubation, O'Meara's reagent(40% KOH+ 0.3% creatine) was added to the tubes. The tubes were mixed properly by shaking. If acetoin is present it will react with the reagent to produce red colour. A positive VP test is indicated by the appearance of a red colour over the surface of the medium within 15-20 minutes of adding reagent. A negative test shows no colour change.

3.7.2.4. Citrate utilization test

Simmon citrate agar(Appendix I)was prepared and was sterilized by autoclaving. The 7ml of the agar was distributed in a sterile test tubes and tubes were kept in a slanting position. Bacterial isolates were streaked on the surface of the Simmon citrate agar slants. Uninoculated slant was kept as control. Slants were incubated at room temperature for 24-48 hours. After incubation, observed for the colour change of the slants. If the bacteria is capable of utilizing citrate, it will metabolize the citrate present in the medium, producing alkaline by-products that raise the pH of

the agar. This causes bromothymol blue indicator to change the colour from green to blue indicating positive result. If there is no colour change, it indicates that the bacteria was unable to utilize citrate as the sole carbon source.

3.7.2.5. Kligler's Iron Test

Triple sugar Iron (Tsin I) agar(Appendix I) slants were inoculated using both streaking and stabbing techniques to assess various fermentation and gas production patterns of the bacterial cultures. The inoculated slants were incubated for 24 hours at room temperature. Following incubation, the colour changes Fermentation in the agar medium were observed and interpreted as follows:

Acid production: A yellow colour change in the medium throughout the slant and butt(deep portion) indicates fermentation of one or more sugars present(glucose, lactose, sucrose).

Sugar Fermentation patterns:

Yellow slant/yellow butt: Fermentation of all sugars(glucose, lactose, sucrose).

Yellow slant/red butt: Fermentation of only glucose.

No fermentation: The red colour of the medium remains unchanged, indicating no sugar fermentation.

Gas production: The presence of bubbles or cracks in the agar medium signifies gas production during fermentation.

Hydrogen Sulfide(H_2S) Production: The blackening of butt of the medium indicates the production of hydrogen sulfide gas(H_2S) by the bacteria, resulting in the formation of iron sulfide precipitates.

3.7.2.6. Urease test

To perform the urease test, the urease agar medium (Appendix I) was prepared aseptically and were distributed in sterile test tubes. The tubes were kept in a slanting position. Slants were streaked with the bacterial isolate. Uninoculated tubes were kept as control. The tubes were incubated at room temperature for 24-48 hours. After incubation tubes were observed for colour change. A positive result is indicated by a change in colour of the medium from yellow to pink which occurs due to the alkaline pH resulting from the hydrolysis of urea and the production of ammonia. A yellow colour indicates a negative result which shows that the bacteria does not possess urease activity.

3.7.2.7. Catalase test

A clean grease free slide was taken and a drop of hydrogen peroxide was transferred on it. Using a sterile inoculating loop, the bacterial isolate was applied on the hydrogen peroxide solution on the slide. Observed for the reaction. Formation of the bubbles indicates the presence of catalase. No bubbles formation indicates the absence of catalase.

3.7.2.8. Motility test

A nutrient broth containing 0.5% agar was prepared aseptically. Using a sterile inoculating loop, the bacterial isolate was taken and stabbed of about half the depth of the medium. Uninoculated tube was kept as control. Tubes were incubated at room temperature for 24 hours. After incubation, the tubes were examined for any diffusion of growth away from the stab line indicating positive result for motility test. No spreading growth or diffusion in a tube indicates negative result.

3.7.2.9. Hugh-Leifson's test

Hugh Leifson medium(Appendix I) was prepared in an aseptic condition. Two sets of tubes were made, in one set of tubes paraffin oil was poured making a layer of 1ml (this will create anaerobic conditions). Other set of tubes were unsealed to allow for aerobic condition. Control tubes were kept for both sets of tubes. Using sterile inoculation loop tubes were inoculated with bacterial isolate and were incubated at room temperature for 24-48 hours. After incubation, tubes were observed for growth and colour changes. A positive carbohydrate utilisation test is indicated by the development of a yellow colour in the medium. A negative test is indicated by the absence of a yellow colour (medium remains green). Development of yellow colour in unsealed tubes were indicated as oxidative while the yellow colour in sealed tubes were indicated as oxidative. Yellow colour in both sealed and unsealed tubes were indicated as oxidative-fermentative.

3.7.2.10. Screening of isolates for acid production by using different sugars

Glucose Phosphate Broth(Appendix) was prepared separately in an aseptic condition. All the sugars including lactose, glucose, sucrose, maltose, xylose and fructose were prepared and autoclaved separately. Each sugar was mixed with the medium 1 and about 5 ml were transferred in sterile test tubes. The bacterial isolate was picked and inoculated into the medium. Tubes were incubated at room temperature for 24-48 hours. After incubation, few drops of methyl red indicator was added in each tube. Tubes were mixed gently by shaking to ensure thorough mixing of the indicator. Observed the colour change of the broth. A red color indicates a positive result that the bacteria produces acidic end products from glucose fermentation. A negative result was indicated by yellow or orange colour.

3.RESULT AND ANALYSIS

4.1. Isolation of cellulose-degrading bacteria

The samples were collected from having high cellulose rich sources. About 37 bacterial isolates were obtained from 9 different samples (Table 1). The colony characters of all the isolates were recorded. These isolates were screened for the industrially important enzymes (cellulase, amylase, xylanase).

Table 4.1: Number of bacterial isolates obtained from each sample

Sample no.	Cellulose rich samples	No. of bacterial isolates
S1	Coconut husk	4
S2	Leaf litter	4
S3	Rice straw	5
S4	Sugarcane bagasse	1
S5	Grass	4
S6	Kitchen waste	8
S7	Garden soil	6
S8	Termites	3
S9	Crushed apple	2

Key:(Letter "S" denotes the sample)

Fig.4.1.1: Isolation of cellulose degrading bacteria from high cellulose content sources in broth

4.2. Purification of isolates

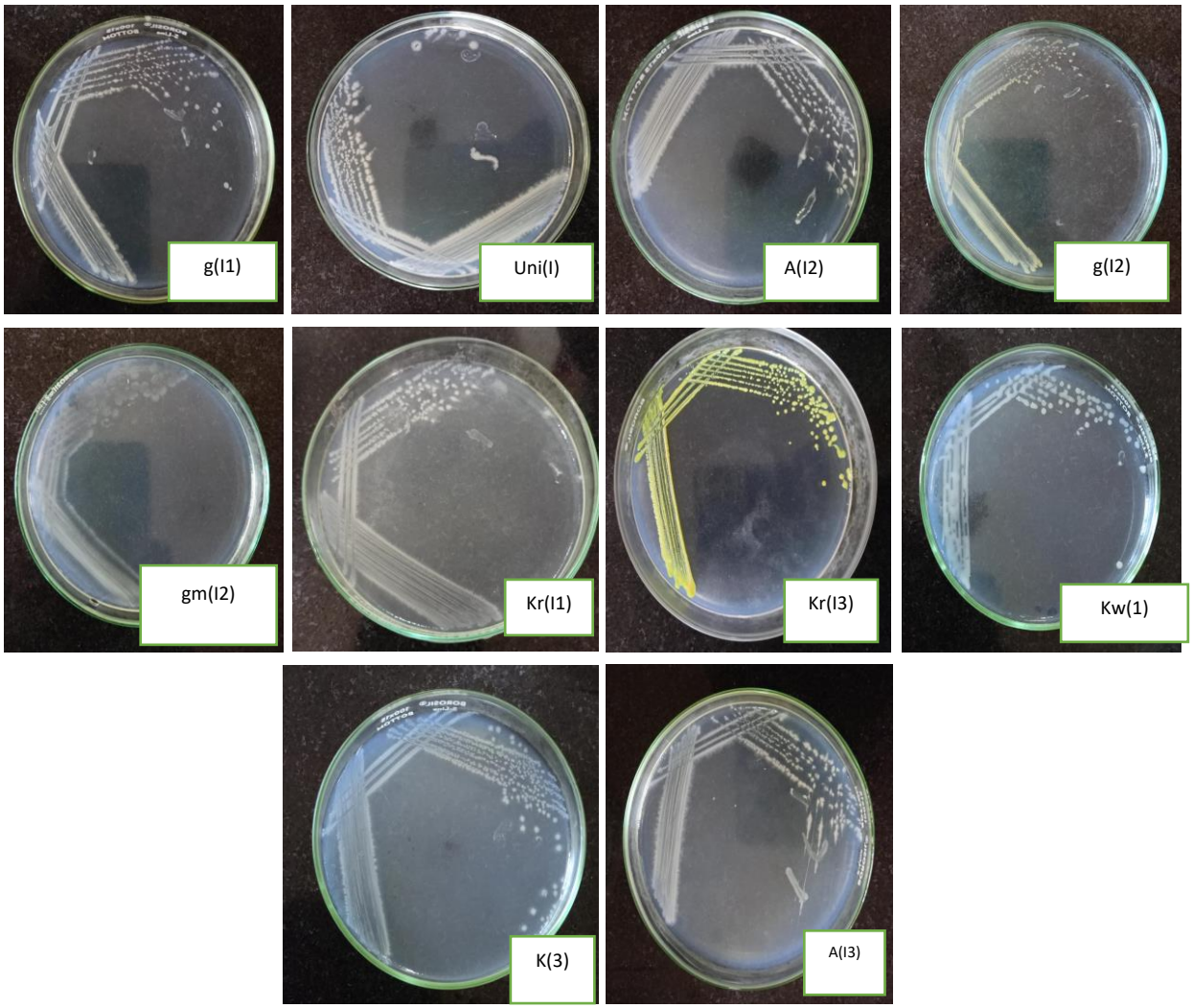

Fig.4.2.1: Purified colonies of bacterial isolates

4.3. Primary screening of cellulase enzyme

4.3.1. Screening of the bacterial isolates for cellulase activity

Thirty-seven isolates were spot inoculated on CMC agar medium. Ten out of thirty-seven bacterial isolates showed a zone of clearance around the colony after the addition of Congo red dye, indicating cellulase production. These (fig 4.3.1.1.)isolates were k(3), kw1, kr(I1), g(I2), g (I1), uni (I), kS (I3), A (I2), A (I3) and gm (I2).

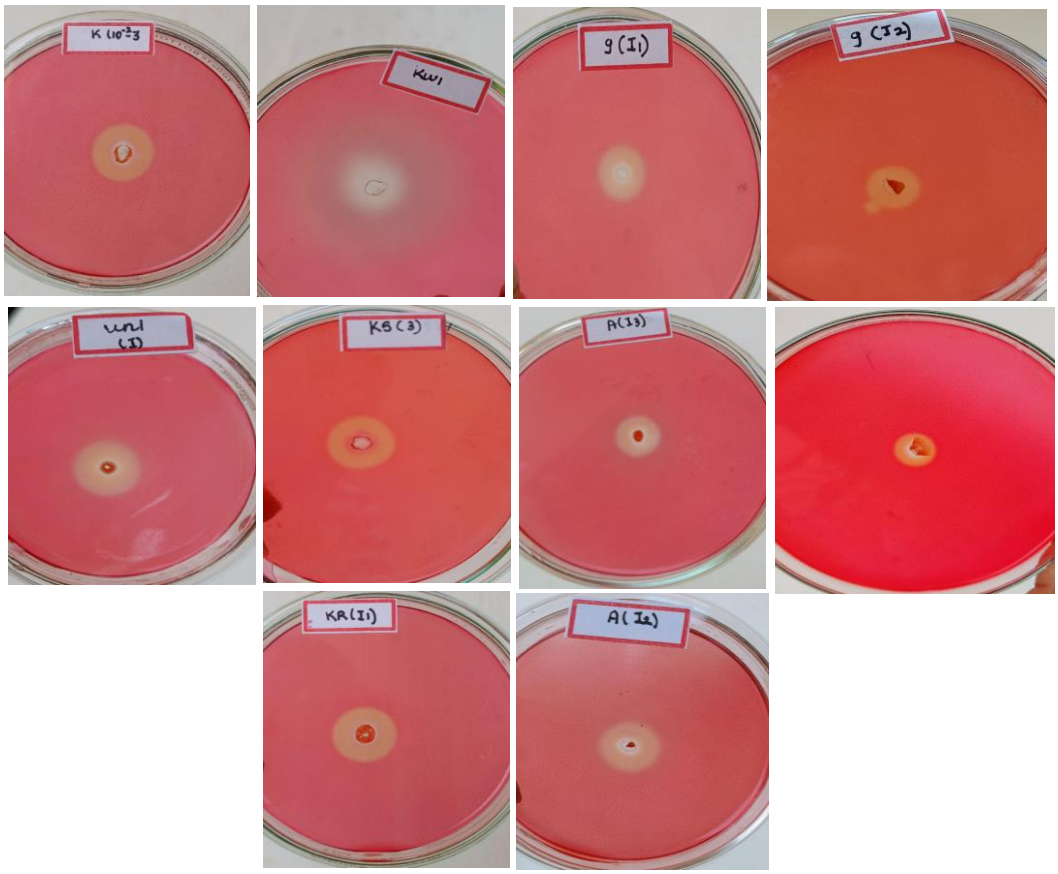

Fig 4.3.1.1: Production of enzyme cellulase by cellulose-degrading bacteria.

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Table 4.3.1: Cellulolytic activity of the bacterial isolates

Isolates	Source of isolates	Diameter of bacterial colony(d) (mm)	Diameter of zone(D) (mm)	Cellulolytic index(D/d) (mm)
k3	Coconut husk	6	20	3.33
kw1	Kitchen waste	6	15	2.50
g(I1)	Garden soil	4	14	3.50
g(I2)	Garden soil	2	12	6.00
uni(I)	Leaf litter	5	17	3.40
kr(3)	Grass	5	18	3.60
A(I3)	Crushed apple	4	15	3.75
gm(I2)	Termites	1	9	9
Kr(I1)	Grass	6	19	3.16
A(I2)	Crushed apple	5	15	3.00

Zone of clearance exhibiting after staining was measured along with the diameter of bacterial colony. The cellulolytic index (Table 4.3.1) was calculated by using a formula HC=D/d where, D indicates diameter of zone of clearance around bacterial colony and d indicates diameter bacterial colony. The data shows significant variation in cellulose degradation among different bacterial isolates. Bacterial isolate 'gm(I2)' shows the highest cellulolytic index of 9.00, indicating cellulose- degrading capabilities. Isolates 'kw(1)' and ' A(I2)' have lower indices of 2.50 and 3.00 respectively.

4.4. Secondary screening of the enzymes

Ten bacterial isolates that tested positive during the primary screening of cellulase were further chosen for secondary screening of enzymes.

4.4.1. Screening for enzyme Amylase

Ten bacterial isolates that were tested positive during screening of cellulase was spot inoculated on the basal medium containing starch as substrate. Fig (4.4.1.1) Seven out of ten bacterial isolates showed zone of clearance after addition of Lugol's iodine, indicating production of amylase. These colonies were (a)g(I1),(b) A(I3)(c), A(I2), K(3), (d)(gm(I2), (e)uni(I) and(f) kr(I1).

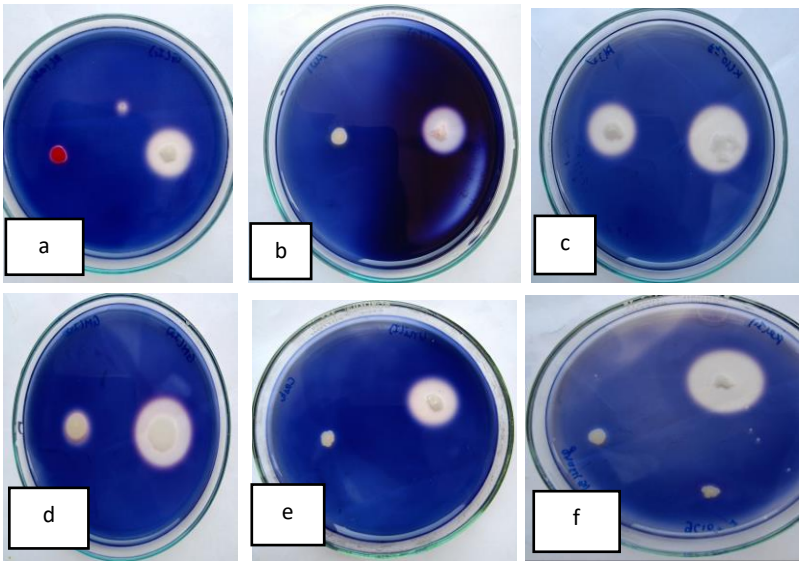


Fig.4.4.1.1: Screening for the production of enzyme amylase

Table 4.4.1: Amylase activity of the bacterial isolates

Isolates	**Source of isolates**	**Diameter of bacterial colony(d) (mm)**	**Diameter of zone(D) (mm)**	**Amylase index(D/d) (mm)**
k3	Coconut husk	7	24	3.42
kw1	Kitchen waste	-	-	-
g(I1)	Garden soil	7	19	2.71
g(I2)	Garden soil	-	-	-
uni(I)	Leaf litter	6	18	3
kr(3)	Grass	-	-	-
A(I3)	Crushed apple	6	20	3.33
gm(I2)	Termites	11	22	2
Kr(I1)	Grass	5	23	4.6
A(I2)	Crushed apple	8	19	2.37

Keys: (-) -No zone of clearance/no growth

Among the bacteria tested,isolate kr(I1) showed the highest level of amylase activity,while isolate gm(I2) showed the least..This indicates that isolate kr(I1) was most efficient in breaking down starch.Other isolates had a moderate range of amylase potential(2-4).

4.5. Screening for enzyme Xylanase

Bacterial isolates were spot inoculated on a basal medium containing xylan as substrate to check for the production of enzyme xylanase. (Fig.4.5.1.) Six out of ten isolates showed yellow opaque zone around the bacterial colony indicating the production of enzyme xylanase. These bacterial isolate includes (a)gm(I2),(b) k(3),uni(I), (c)A(I3), (d)A(I2) and kr(I1).

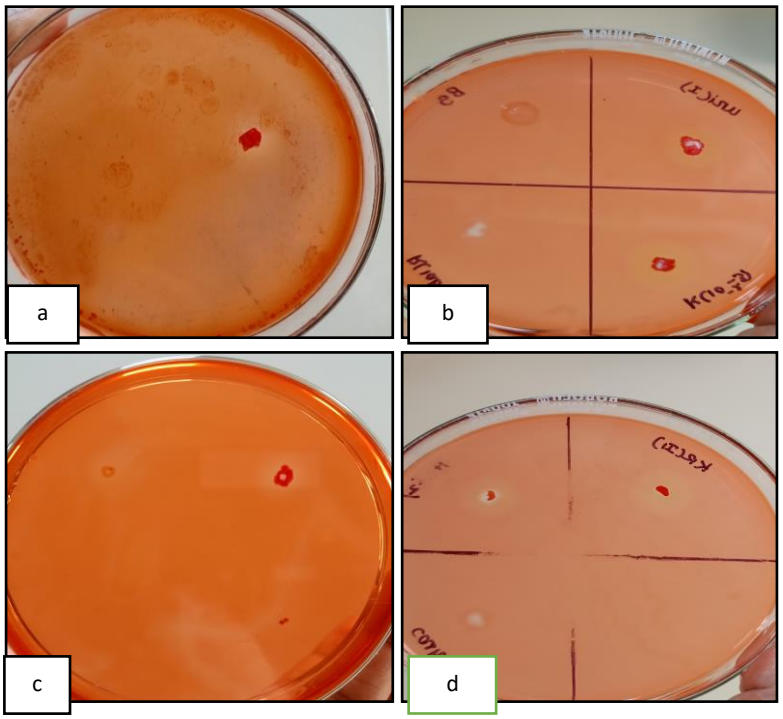


Fig.4.5.1: Production of enzyme xylanase

Table 4.5.1: Xylanase activity of the bacterial isolates

Isolates	**Source of isolates**	**Diameter of bacterial colony(d) (mm)**	**Diameter of zone(D) (mm)**	**Cellulolytic index(D/d) (mm)**
k3	Coconut husk	4	15	3.75
kw1	Kitchen waste	-	-	-
g(I1)	Garden soil	-	-	-
g(I2)	Garden soil	-	-	-
uni(I)	Leaf litter	3	13	4.33
kr(3)	Grass	-	-	-
A(I3)	Crushed apple	4	7	1.75
gm(I2)	Termites	5	7	1.4
Kr(I1)	Grass	3	14	4.66
A(I2)	Crushed apple	2	13	6.5

Keys: (-) -No zone of clearance/no growth

Among these ten isolates,six were found to be efficient in xylan hydrolysis.Isolate kr(I1) showed the highest level of xylanase activity,while isolate gm(I2) showed the least.

Due to their combined abilities of hydrolyzing

- CMC(Cellulase activity)
- Starch(Amylase activity)
- Xylan(Xylanase activity)

  These isolates can become a strong candidate for forming consortium(mixed group of microorganisms).Every isolate contributes unique enzyme capabilities,enhancing the efficiency of consortium in decomposing various components of farm residues.

4.6. Screening of isolates for acid production

Methyl red test used to differentiate between the bacteria that are acid producers and non-acid producers.(Fig.4.6.1.),out of ten, seven bacterial isolates were found to be acid producers(indicating red colour after addition of methyl red indicator).Isolates showing positive result for methyl red test were (a) uni(I) (b) A(I2) (c) A(I3) (d) kr(I1) (e) kw(1) (f) k(3) and (g) g(I1).Negative result were indicated by a yellow colour, indicating non-acid producers.

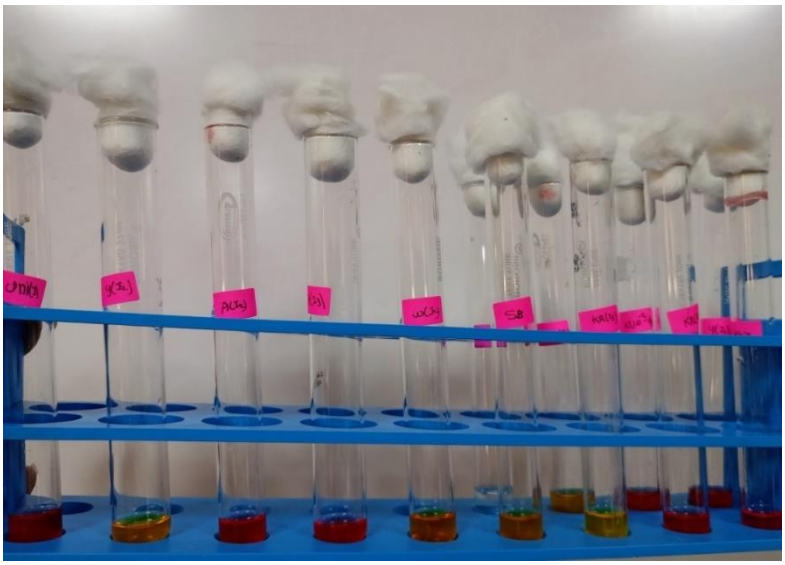


Fig.4.6.1:Acid production by bacterial isolates

4.7. Morphological characterization

4.7.1. Gram staining

Gram staining of the bacterial isolates showed two different cells: purple (Gram-positive) and pink (Gram negative).Among ten isolates,3 isolates were found to be Gram negative and 7 isolates were found to be Gram positive.All isolates were found to be rod-shaped.

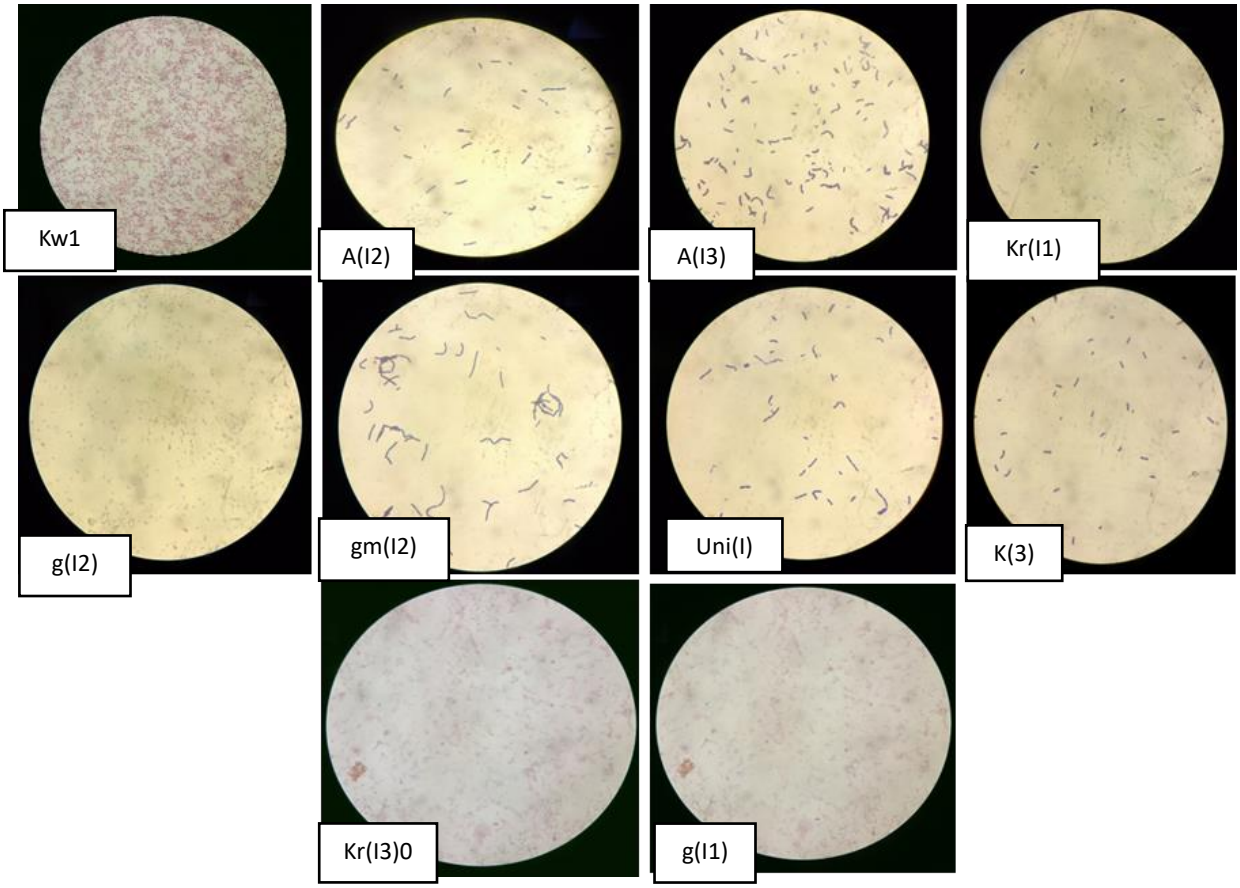


Fig.4.7.1: Visualization of bacterial morphology

Table 4.7.1: Colony characteristics of the bacterial isolates

Isolate	**Kw(1)**	**g(I1)**	**A(I2)**	**A(I3)**	**G(I2)**
Source	Kitchen Waste	Garden Soil	Crushed Apple	Crushed Apple	Garden Soil
Shape	Round	Round	Round	Spindle	Irregular
Size	0.3 Cm	0.2 Cm	0.2 Cm	0.5 Cm	0.4 Cm
Surface	Smooth	Smooth	Smooth	Smooth	Dull
Colour	White	White	White	White	Yellow
Opacity	Opaque	Opaque	Opaque	Opaque	Translucent
Elevation	Convex	Flat	Flat	Flat	Flat
Margin	Entire	Entire	Entire	Entire	Entire
Consistency	Slimy	Moist	Moist	Moist	Butyrous
Gram Character	Gram Negative Rods	Gram Negative Rods	Gram Positive Rods	Gram Positive Rods	Gram Positive Rods

Table 4.7.2: Colony characteristics of the bacterial isolates

Isolates	**K(3)**	**Kr(I1)**	**Uni(I)**	**Kr(I3)**	**gm(I2)**
Shape	Round	Spindle	Round	Round	Wavy
Size	0.4 Cm	0.3 Cm	0.4 Cm	0.3 Cm	0.4 Cm
Surface	Smooth	Smooth	Smooth	Lustrous	Dull
Colour	White	White	White	Yellow	White
Opacity	Opaque	Opaque	Opaque	Opaque	Translucent
Elevation	Flat	Flat	Flat	Convex	Flat
Margin	Entire	Entire	Entire	Entire	Lobate
Consistency	Moist	Moist	Moist	Slimy	Dry
Gram Character	Gram Positive Rods	Gram Positive Rods	Gram Positive Rods	Gram Negative Rods	Gram Positive Rods

4.8. Biochemical test results

4.8.1. Indole test

Indole test was performed to check the ability of the bacterial isolates to convert tryptophan into indole. A positive result is indicated by the formation of red colour ring on the surface of the medium. A negative result is indicated by no formation of red colour ring over the surface of medium. Indole tests performed shows negative result for all the bacterial isolates

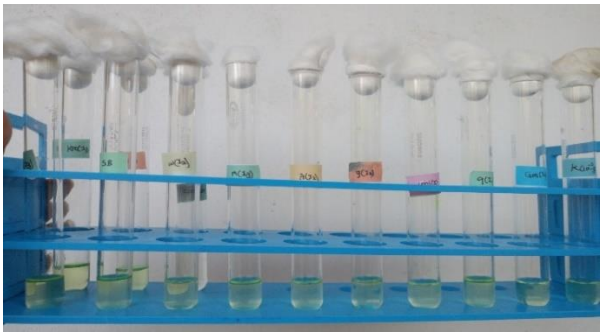


Fig.4.8.1: Indole tests for the bacterial isolates

4.8.2. Methyl red test

Methyl red test was used to determine the acidity of a solution. Methyl red indicator was added to the broth to test the acid producing isolates. In this test, seven bacterial isolates were tested positive which were indicated by red colour of the solution. Formation of a yellow colour indicates negative result for methyl red test.

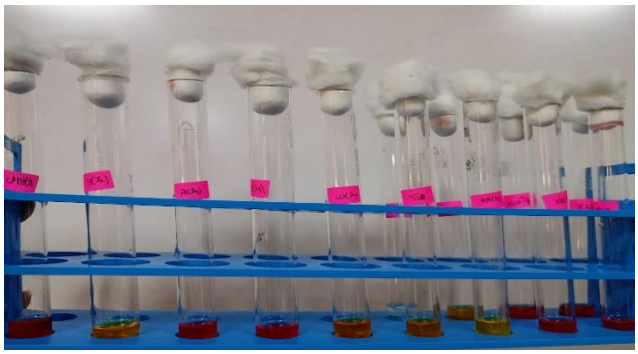


Fig 4.8.2. Acid production by isolates

4.8.3 Voges-Proskauer test

The Voges-Proskauer test detects the presence of acetoin in the medium. The test was performed by adding Omeara's reagent and observed for the formation of red colour in the medium indicating a positive result. No colour change indicates a negative result. The test performed shows negative results for all the bacterial isolates.

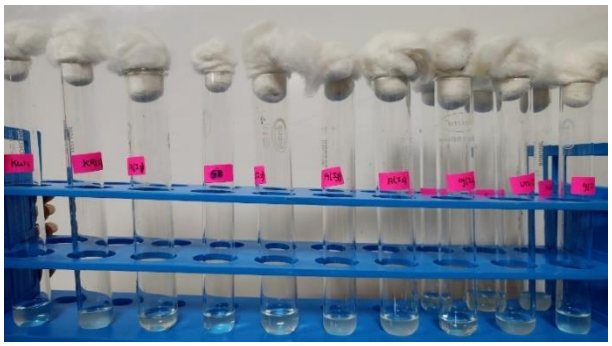


Fig 4.8.3 Detection of presence of acetoin of isolates by Voges- Proskauer test

4.8.4 Citrate utilization test

The test was performed on Simmon citrate agar slants. A positive test result was indicated by a blue coloration in the medium while negative result was indicated by no colour change in the tube. Two bacterial isolates kw(1) and kr(I3) showed positive result indicating blue colouration.

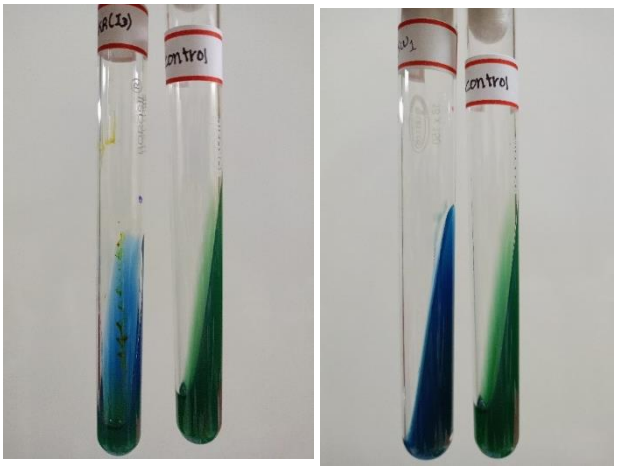


Fig 4.8.4: Characterisation of isolates by citrate utilization test

4.9. Kligler's iron test

Kligler's iron test was performed to evaluate the ability of the organism to ferment glucose lactose and sucrose as well as to check the ability of the bacterial isolates for the production of hydrogen sulfide(H2S) and gas. The result of the TSI test was tabulated in table.4.9.1

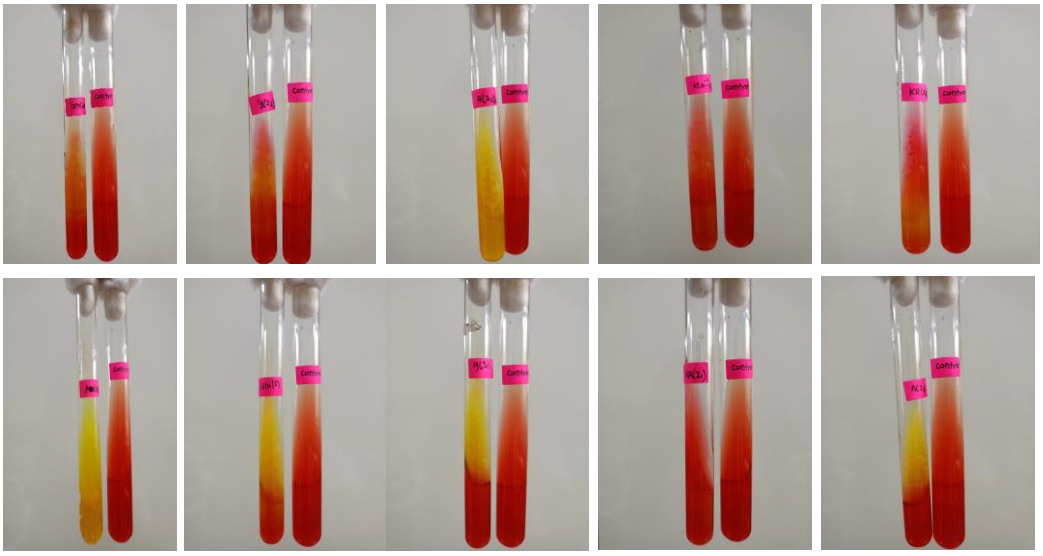


Fig 4.9.1. Visual representation of Kligler's test

Table 4.9.1: Kligler's test

Isolates	**Slant**	**Butt**	**Gas**	**H_2S**
gm(I2)	A	K	-	-
g(I2)	A	K	-	-
A(I2)	A	A	-	-
k(3)	K	K	-	-
kr(I3)	K	A	-	-
kw(1)	A	A	+	-
g(I1)	A	K	-	-
kr(I1)	K	K	-	-
A(I3)	A	K	-	-
uni(I1)	A	K	-	-

The bacterial isolates were observed for the colour change in the slant and butt, gas production (bubbles or cracks) and hydrogen sulfide production (black precipitate). (A) in the table indicates acidic butt/slant;(K) indicates Alkaline slant/Butt. Gas production and hydrogen sulfide production was indicated by positive(+) sign and no gas or H2S production was indicated by negative(-) sign.

4.10. Urease test

The urease test was performed and was observed for the colour change in the medium. The change in the medium from yellow to pink indicates the positive result. No colour change of the medium indicates that the test is negative. Four bacterial isolates (a) gm(I2),(b) k(3),(c) kr(I1) and (d) kr(I3) were found positive for the test performed indicating pink colouration of the medium which shows the bacteria present in the medium can hydrolyze urea.

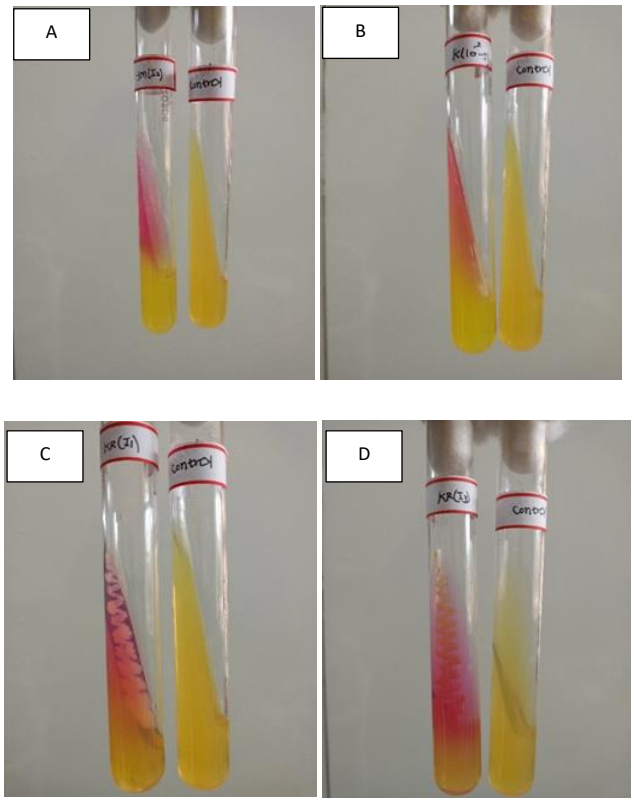


Fig 4.10: Detection of Urease enzyme by isolates

4.11. Catalase test

The catalase test was used to determine the presence of the enzyme catalase in the bacterial isolates. Formation of bubbles after addition of hydrogen peroxide indicates positive test. All the bacterial isolates including A(I3),A(I2),g(I1),uni(I),kr9I1),g(I2),kr(I3),k(3),gm(I2) were found positive while isolate kw(1)was found to be negative for catalase test.

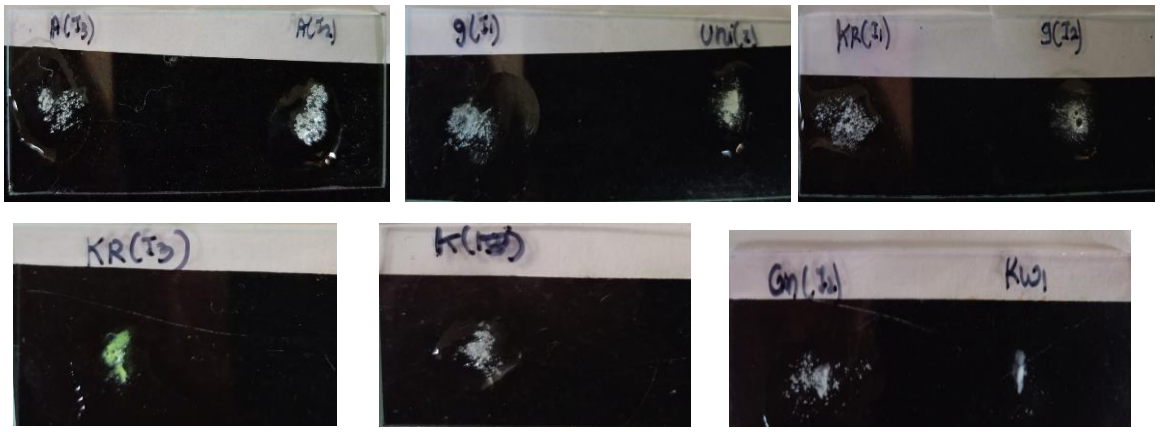


Fig 14: Catalase test

4.12. Motility test

Motility test assessed the ability of the bacterial isolate to move actively. Fig: A shows that the five bacterial isolates were observed for the growth radiating out from the stab line indicates that the organisms are motile. Fig: B shows no growth beyond the stab line, indicating that the organisms are non-motile.

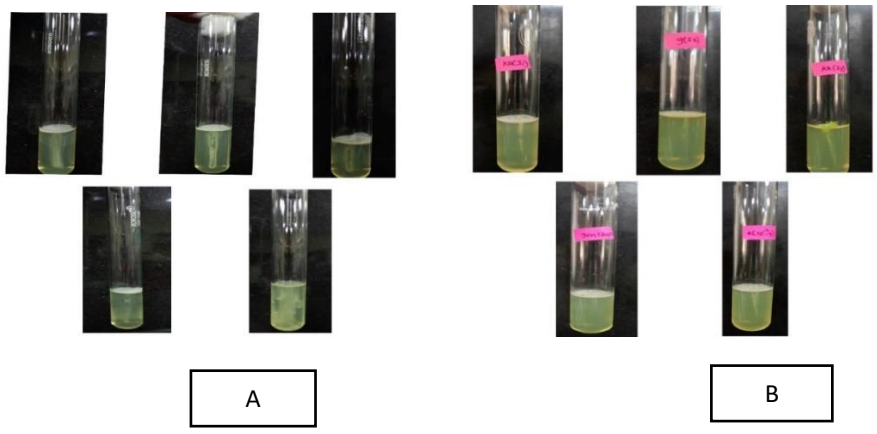
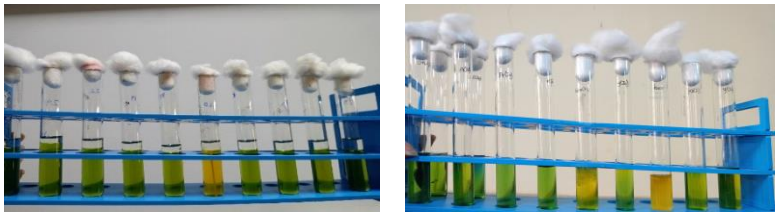


Fig 4.12.1. Visual representation of motility test

4.13. Hugh leifson test

This test is used differentiate bacteria based on the ability to utilize glucose in the presence of oxygen. Results are interpreted based on growth and colour changes in both open and closed tubes. Positive oxidative bacteria is indicated by growth and colour change in open tube while positive fermentative is indicated by colour change in closed tubes. A positive oxidative-fermentative test is shown by colour change in both open and closed tubes.

In this test, isolate kw(1) has was found positive for both oxidative and fermentative tubes while isolate gm(I2) was found to be positive oxidative.



A B

Fig.4.13.1. Visual representation of Hugh Leifson

4.14. Screening for acid production

This test was performed to check the ability of the bacterial isolates for acid production using different sugars. Methyl red indicator was used to check for acid production. Among the sugars, maltose exhibits highest acid production followed by sucrose, lactose, xylose etc.Results are tabulated in table 4.14.1.

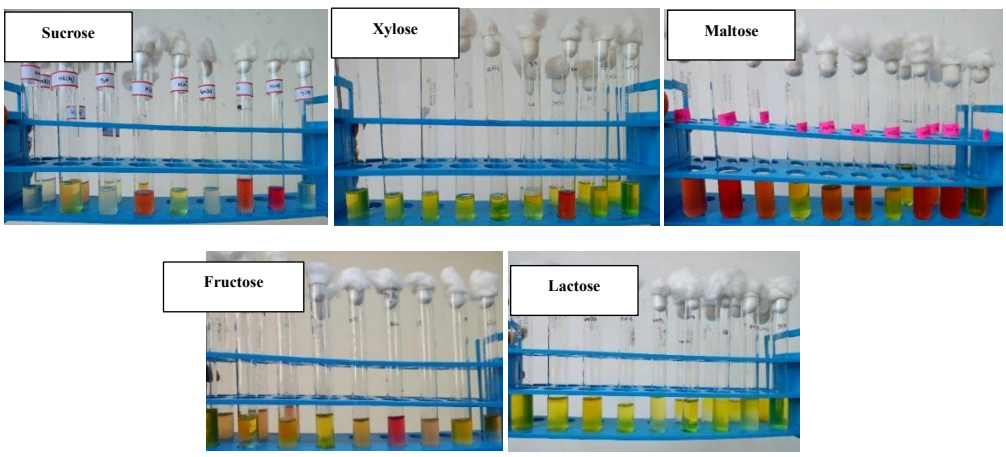


Fig 4.14.1: Screening of bacterial isolates for acid production using different sugars

Table 4.14.1.: Biochemical test results

Biochemical tests were performed for the selected ten isolates and the results were tabulated as shown in table.

Isolates	**kw(1)**	**g(I1)**	**A(I2)**	**A(I3)**	**g(I2)**
Indole	-	-	-	-	-
Methyl Red	+	+	+	+	-
Voges-Proskauer	-	-	-	-	-
Citrate test	+	-	-	-	-
Catalase test	-	+	+	+	+
Motility test	+	+	+	+	-
Urease test	-	-	-	-	-
Hugh leifson					
Aerobic	+	-	-	-	-
Anaerobic	+	-	-	-	-
Carbohydrate utilization test					
Lactose	-	-	-	-	-
Sucrose	+	+	+	+	-
Maltose	+	+	+	+	-
Fructose	+	-	-	-	-
Xylose	+	-	-	-	-

Table 4.14.2: Biochemical test results

Biochemical test	k(3)	kr(I1)	kr(I3)	uni(I)	gm(I2)
Indole	-	-	-	-	-
Methyl Red	+	+	-	+	-
Voges-Proskauer	-	-	-	-	-
Citrate test	-	-	+	-	-
Catalse test	+	+	+	+	+
Motility test	-	-	-	+	-
Urease test	+	+	+	-	+
Hugh leifson					
Aerobic	-	-	-	-	-
Anaerobic	-	-	-	-	-
Carbohydrate utilization test					
Lactose	-	-	-	-	-
Sucrose	-	-	-	-	-
Maltose	+	+	+	+	-
Fructose	-	-	-	-	-
Xylose	-	-	-	-	-

Key: (+): positive; (-): negative

In this study, bacterial isolates were screened for industrially important enzymes where some of them showed the efficient ability to degrade the components of farm residues such as cellulose, starch and xylan. Isolates having such potential in producing enzymes can be beneficial for various purposes such as in waste management and in biofuel production. These findings serve as a foundation for the formation of microbial consortia, as they suggest the existence of complementary enzymatic activities among the isolates. Based on these findings, we anticipate achieving greater enzymatic diversity and substrate specificity. This is expected to improve the overall effectiveness and versatility of these microbes together across a range of biotechnological applications.

FUTURE PROSPECTS

Development of a potent bacterial consortium by combining the most promising isolates. Thus, aiming to degrade a wide range of farm residues.

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APPENDIX I

MEDIA COMPOSITION

1) Basal medium

Ingredients	g/litre
Potassium dihydrogen phosphate	1.36
Magnesium sulphate heptahydrate	0.20
Sodium cholride	2.00
Ferrous sulphate heptahydrate	0.01
Carboxymethyl cellulose	3.00
Yeast extract	1.00
Agar	15.00
pH	6.8-7.2

23.57 g is suspended in 1000 ml distilled water.Dissolve the media completely by heating.

Autoclave at 15 lbs pressure(121^0C) for 15 minutes.Mix well and pour in sterile petri plates.

2)Basal media broth

Ingredients	g/litre
Potassium dihydrogen phosphate	1.36
Magnesium sulphate heptahydrate	0.20
Sodium cholride	2.00
Ferrous sulphate heptahydrate	0.01
Carboxymethyl cellulose	3.00
Yeast extract	1.00
Agar	15.00
pH	6.8-7.2

3) Modified basal medium(1% CMC-Carboxymethyl cellulose)

Ingredients	g/litre
Potassium dihydrogen phosphate	1.36
Magnesium sulphate heptahydrate	0.20
Sodium cholride	2.00
Ammonium sulphate	1.00
Ferrous sulphate heptahydrate	0.01
Agar	15.00
CMC	10.00
Yeast extract	1.00
pH	6.8-7.2

4) Modified basal medium(1% xylan)

Ingredients	g/litre
Potassium dihydrogen phosphate	1.36
Magnesium sulphate heptahydrate	0.20
Sodium cholride	2.00
Ammonium sulphate	1.00
Ferrous sulphate heptahydrate	0.01
Agar	15.00
Xylan	10.00
Yeast extract	1.00
pH	6.8-7.2

5) Modified basal medium(1% Starch)

Ingredients	g/litre
Potassium dihydrogen phosphate	1.36
Magnesium sulphate heptahydrate	0.20
Sodium cholride	2.00
Ammonium sulphate	1.00
Ferrous sulphate heptahydrate	0.01
Agar	15.00
Starch	10.00
Yeast extract	1.00
pH	6.8-7.2

BIOCHEMICAL MEDIUM COMPOSITION

1)Glucose Phoshate Broth(MR-VP Medium)

Ingredients	g/litre
Peptone	7.0
Dipotassium phosphate	5.0
Glucose	5.0
Sodium chloride	1.4
pH	7.2+/-0.2

2) Sugar stock solution

Ingredients	G/litre
Sugar (Lactose/sucrose/maltose/fructose/xylose)	5.0
Distilled water	1L

Sugars were prepared as 0.5% (w/v) 10 ml stock solutions, one for each sugar. These solutions were intended for use in the methyl red test. Each solution was autoclaved separately for 10 minutes at 121°C.

3)Tryptone broth

Ingredients	g/litre
Tryptone	10.00
Nacl	5.00
Distilled water	1L

4)Christensen's Urea medium

Ingredients	g/litre
Peptone	1.0
KH2PO4	2.0
NaCl	5.0
Urea	20.0
Phenol red	0.1
Distilled water	1 l
pH	6.8

Urea was added separately to the medium, as it is prone to degradation during autoclaving. 20% urea stock solution was prepared and sterilized by filter sterilization

method. The appropriate volume of urea stock was added to the above medium broth, after it has been sterilized by autoclaving at 121°C for 20 minutes.

5) Simmons Citrate agar

Ingredients	g/litre
Nacl	1.0
Mgso4.7H2O	0.2
Nahpo4	1.0
Kh2po4	1.0
Sodium citrate	1.0
Bromothymol blue	9.0
Agar	8.0
Distilled water	1 L
Ph	6.8

6) Hugh Leifson medium

Ingredients	g/litre
Peptone	**2.0**
Sodium chloride	**5.0**
Dipotassium phosphate	**0.3**
Glucose	**10.0**
Bromothymol blue	**0.03**
Agar	**3.0**
pH	**7.1**

APPENDIX II

REAGENTS AND STAINS

1)1% Lugol's iodine solution

Ingredients	g/100ml
Lugol's iodine	1 ml
Distilled water	100 ml

2) 0.1% Congo Red Solution

Ingredients	g/100 ml
Congo red	0.1
Distilled water	100 ml

3) 1M NaCl solution(for destaining)

Ingredients	g/100 ml
NaCl	24.72
Distilled Water	100 ml

4) 2% Saline

Ingredients	g/litre
NaCl	**2**
Distilled Water	**100 ml**

5) Methyl red reagent(for MR test)

Ingredients	g/400 ml
Methyl red	6.2
Ethyl alcohol	600 ml
Distilled water	400 ml

6) Kovac's reagent(Indole test)

Ingredients	g/litre
Isoamyl alcohol	150 ml
p-dimethyl amino benzaldehyde	10.0
Concentrated HCL	50 ml
Distilled Water	1000 ml

7) Omeara's reagent(Voges-Proskauer test)

Ingredients	g/40 ml
Creatine	0.15
KOH	20.0
Distilled water	40 ml

8) Gram's Crystal Violet

Ingredients	g/80 ml
Crystal Violet	2.0
Ammonium oxalate	0.8
Ethyl alcohol	20 ml
Distilled water	80 ml

9) Safranine

Ingredients	g/300 ml
Safranine-O	0.5
Ethyl alcohol	50 ml

10) Gram's Iodine

Ingredients	g/300 ml
Iodine	1.0
Potassium iodide	2.0
Distilled water	300 ml